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Sequence and Expression Differences Underlie Functional Specialization of *Arabidopsis* MicroRNAs miR159 and miR319

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SUMMARY

Many microRNAs (miRNAs) are encoded by small gene families. In a third of all conserved *Arabidopsis* miRNA families, members vary at two or more nucleotide positions. We have focused on the related miR159 and miR319 families, which share sequence identity at 17 of 21 nucleotides, yet affect different developmental processes through distinct targets. MiR159 regulates *MYB* mRNAs, while miR319 predominantly acts on *TCP* mRNAs. In the case of miR319, *MYB* targeting plays at most a minor role because miR319 expression levels and domain limit its ability to affect *MYB* mRNAs. In contrast, in the case of miR159, the miRNA sequence prevents effective *TCP* targeting. We complement these observations by identifying nucleotide positions relevant for miRNA activity with mutants recovered from a suppressor screen. Together, our findings reveal that functional specialization of miR159 and miR319 is achieved through both expression and sequence differences.

INTRODUCTION

MicroRNAs (miRNAs) are short, noncoding RNAs, about 21 nucleotides in length, with variable sequence complementarity to longer target RNAs. The typical target mRNA in animals has multiple motifs with limited sequence complementarity to one or more miRNAs in its 3′ untranslated region (UTR), and the predominant mode of miRNA action is translation inhibition or deadenylation-mediated mRNA degradation. In contrast, the typical target mRNA in plants has a single miRNA-complementary motif in the coding region with few or no mismatches, and the principal mode of action is transcript cleavage. Plant miRNAs have therefore often been compared to perfectly complementary short interfering RNAs (siRNAs) (Bartel, 2004; Filipowicz, 2005; Jones-Rhoades et al., 2006). The differences between animal and plant miRNAs are, however, not absolute. Human miR196 directs cleavage (Yekta et al., 2004), and at least one plant miRNA, miR172, acts both by preventing productive translation and by target cleavage (Aukerman and Sakai, 2003; Chen, 2004; Kasschau et al., 2003; Lauter et al., 2005; Schwab et al., 2005).

MiRNAs are processed from larger precursors containing a self-complementary fold-back structure. Mature miRNAs are incorporated into RNA induced silencing complexes (RISCs) that include members of the ARGONAUTE (AGO) family of proteins. AGO proteins catalyze ribonucleolytic cleavage of the target at the position opposite of nucleotide 10 of the small RNA (Filipowicz, 2005; Kim, 2005).

The 5′ portions of miRNAs and siRNAs are particularly important for guide function (Doench et al., 2003; Lewis et al., 2003; Mallory et al., 2004b; Parizotto et al., 2004; Vaucheret et al., 2004). In animals, part of the 5′ portion is called the "seed region," and sequence complementarity to this region is often sufficient for target recognition (Brennecke et al., 2005; Lewis et al., 2005). The importance of the 5′ region applies also to plant miRNAs. For example, introduction of mismatches between positions 3 and 11 drastically reduces miRNA-guided cleavage of *PHABULOSA* (*PHB*) mRNA by miR165, with mutations in the 3′ region having much weaker effects (Mallory et al., 2004b). These observations are consistent with global rules of miRNA target interaction that have been experimentally deduced from transcript profiling of plants overexpressing natural or artificial miRNAs (Schwab et al., 2006; Schwab et al., 2005).

Table 1. miR159/miR319 Families

miRNA	Sequence	Times Cloned[a]	Overexpression Phenotype[b]		
			Cotyledon Epinasty	Crinkled Leaves	Stamen Defects
miR159a	.uUUGGAuUGAAGGGAGCUCua	19,940 (4,306)	-	-	~48%
miR159b	.uUUGGAuUGAAGGGAGCUCuu	1,982 (387)	-	-	~46%
miR159c	.uUUGGAuUGAAGGGAGCUCcu	21 (3)	-	-	-
miR319a	..UUGGAcUGAAGGGAGCUCcc/ ..UUGGAcUGAAGGGAGCUCccu	6 (0)/145 (45)	Severe (>90%)	Strong (>90% of T_1)	~33%
miR319b			Severe (>90%)	Strong (>80% of T_1)	Not determined
miR319c	.uUUGGAcUGAAGGGAGCUCcu/ ..UUGGAcUGAAGGGAGCUCcuu	12 (3)/17 (8)	Moderate (>50%)	Mostly Normal	~24%

[a] http://asrp.cgrb.oregonstate.edu/; total numbers from all genotypes and tissues; small RNAs unambiguously assigned to wild-type given in parentheses.
[b] At least 80 T_1 plants per construct.

Many plant miRNAs are encoded by gene families, which can have more than a dozen members. Some miRNAs originating from different family members are identical, while others differ in up to three nucleotides, typically near the 3′ end of the mature miRNA. Whether these differences reflect functional specialization of miRNA family members is not known. The *Arabidopsis* genome contains several genes that can potentially produce five distinct miRNAs belonging to the miR159 and miR319 families, which share 17 identical nucleotides but have 5′ ends that are offset by a single nucleotide (Palatnik et al., 2003; Reinhart et al., 2002; Sunkar and Zhu, 2004). The effects of overexpression, as well as the position of target cleavage, have indicated that miR159a and miR319a (also known as miR-JAW) have largely nonoverlapping effects in vivo. MiR159 targets several *MYB* transcription factor genes involved in flowering and male fertility, while miR319 primarily affects a clade of *TCP* transcription factor genes controlling leaf shape (Achard et al., 2004; Millar and Gubler, 2005; Palatnik et al., 2003; Schwab et al., 2005). We have analyzed the basis for the different biological effects of miR159 and miR319, and we show with several approaches that both differences in sequence and expression contribute to specific interactions between these two miRNAs and their targets.

RESULTS

MiR159/miR319 miRNA Families and Their Targets

Mobility of miR319a/b during gel electrophoresis, as well as cloning of miR319c, had suggested that these miRNAs are predominantly 20 nucleotides in length (Palatnik et al., 2003; Sunkar and Zhu, 2004). More recent deep sequencing efforts have shown that, as with other miRNAs, there is some heterogeneity in size, but the 21-nucleotide-long variants appear to dominate (Table 1; http://asrp.cgrb.oregonstate.edu/) (Fahlgren et al., 2007; Rajagopalan et al., 2006). MiR159 and miR319 can potentially be generated from six different precursors. All of the mature miRNAs have been identified in small RNA libraries, although at very different levels (Table 1). The fold-back structures of miR159 and miR319 precursors have similar lengths, and both show sequence conservation in other species outside of the miRNA/miRNA* base-paired region. In addition, the miRNAs all arise from an equivalent position within the 3′ arm of the fold-back (see Figure S1 in the Supplemental Data available with this article online). While precursors from both families are more similar to each other than the average random pair of miRNA precursors, both by sequence and structural criteria (Figure S2), their similarity is not exceptionally high. Thus, though they are often treated as a single family (Jones-Rhoades and Bartel, 2004), it remains unclear whether miR159 and miR319 originated from a common ancestor.

Two groups of miR159/miR319 targets have previously been experimentally validated (Figures 1A and 1B). These include several members of the *GAMYB*-related clade of *MYB* transcription factor genes, which have important roles in hormone response and male fertility, and five *TCP* genes related to *CINCINNATA* from snapdragon, which control leaf growth (Achard et al., 2004; Millar and Gubler, 2005; Nath et al., 2003; Palatnik et al., 2003; Schwab et al., 2005). A third group of targets comprises the recently described *DUO1/MYB125* gene, which has an essential function in the male germline and which defines a unique *MYB* clade in *Arabidopsis* (Rotman et al., 2005). We confirmed that mRNAs encoding *DUO1* as well as *MYB101*, a member of the *GAMYB* clade, are cleaved in wild-type plants in a position that indicates targeting by miR159 (Figure 1C) (Reyes and Chua, 2007). MiR159 regulation of *DUO1* is likely important for its normal function, as shown by overexpression of a form with synonymous mutations that disrupt the miRNA target site (Figure S3).

Cross-Regulation of miR159 and miR319 Targets

Computational predictions using experimentally established criteria (Schwab et al., 2005) suggest that miR319 can target five *TCP* genes but also several *MYB* genes. In contrast, miR159a and miR159b should be specific for *MYB* genes (Figure 1D). Only miR159c would be predicted to target two of five *TCP* genes, but its expression

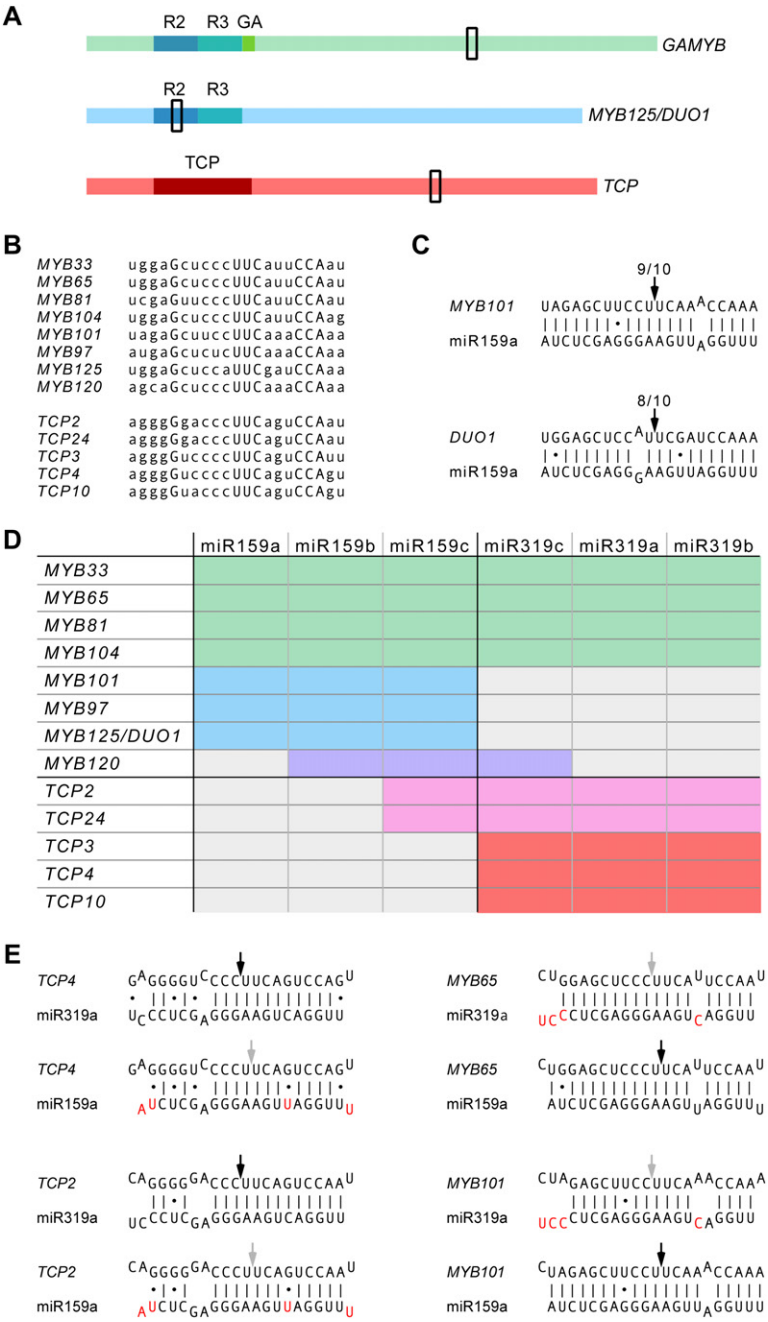

Figure 1. Targets of miR159 and miR319 miRNAs

(A) Diagram of the three different classes of targets. Conserved domains are indicated, and the miRNA target sites are boxed.

(B) Alignment of miRNA targeting sites.

(C) Newly validated miR159 targets. See Figure S3 for additional information on *MYB125/DUO1*.

(D) Predicted targeting of miRNAs based on previously established rules (http://wmd.weigelworld.org) (Schwab et al., 2006; Schwab et al., 2005). Colored boxes indicate targets predicted to be regulated by the same miRNAs. For *MYB125/DUO1*, the unusual G:U pair at position 8 was treated as a perfect match. See Table S1 for further details on miR159/miR319 targets.

(E) Alignments of selected miRNA target duplices. The predominant cleavage sites of the targets found in wild-type are indicated by black arrows, from which the miRNA acting in vivo on a specific target can be deduced. For *MYB65*, 1 out of 13 cleavage products had a 5′ end indicative of targeting by miR319 (Palatnik et al., 2003). In none of the other cases were alternative cleavage sites, indicated by gray arrows, found. Sequence differences between miR319 and miR159 are indicated in red.

levels are two to three orders of magnitude lower than those of miR159a and miR159b.

In an alignment, the 5′ ends of miR159a and miR159b are offset by one nucleotide relative to miR319a/b. Because AGO catalyzes cleavage of targets invariably opposite of the bond between nucleotides 10 and 11 from the 5′ end of the miRNA, cleavage products will differ by one nucleotide depending on which miRNA guides the processing event (Figure 1E). Cleavage site mapping and miRNA overexpression have indicated that the *MYB* genes are predominantly targeted by miR159 in vivo, while *TCP* genes are targeted by miR319a/b (Palatnik et al., 2003; Schwab et al., 2005) (Figure 1E). Overexpression of the endogenous *MIR319a* gene in *jaw*-D mutants leads to epinastic cotyledons and crinkly leaves (Palatnik et al., 2003). We found that plants overexpressing miR319a from the cauliflower mosaic virus 35S promoter had cotyledon and leaf phenotypes similar to those of *jaw*-D mutants, but some 35S:*miR319a* plants had additional stamen defects resembling those of 35S:*miR159a* plants, which are male sterile due to inactivation of *MYB33* and *MYB65* (Achard et al., 2004; Millar and Gubler, 2005) (Figures 2A and 2B). They also had stronger leaf defects than *jaw*-D mutants, suggesting that the stamen defects were due

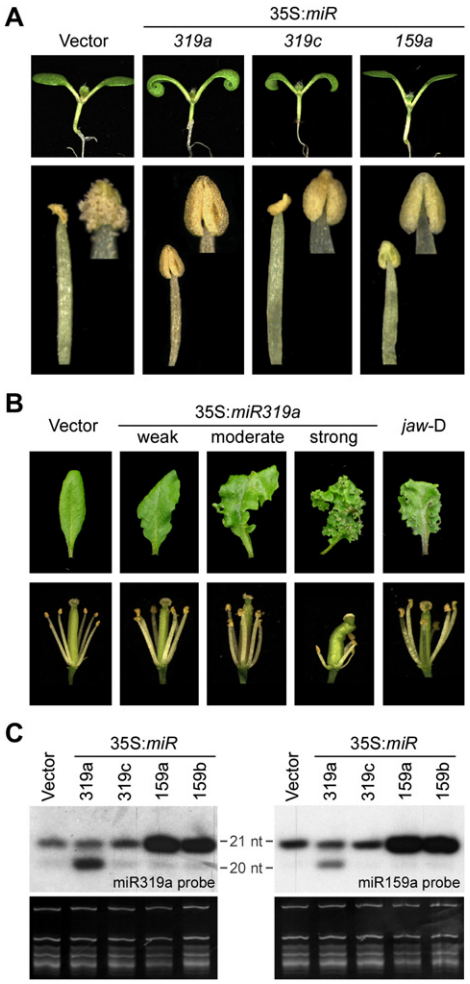

Figure 2. Overexpression of Members of the miR159/miR319 Family

(A) Cotyledon and stamen defects in miR319 and miR159 overexpressers.

(B) Spectrum of leaf and stamen phenotypes in plants that overexpress miR319a, either from the 35S promoter or due to an activation tagging event at the endogenous locus (*jaw*-D).

(C) Small RNA blots of miRNA overexpressers.

to particularly high levels of miR319a in these plants (Figure 2B). Although the majority of 35S:*miR319c* plants had only weakly epinastic cotyledons and relatively normal leaves, many had stamen defects (Figure 2A). MiR319c accumulated to moderate levels in these plants (Figure 2C), and it is possible that higher levels may have resulted in crinkled leaves as well. Thus, strong overexpression of miR319a or moderate overexpression of miR319c causes a male sterility phenotype similar to that seen in miR159 overexpressers.

We previously analyzed 22 *MYB33* and *MYB65* cleavage products and found that the 5′ end of only one *MYB65* product was compatible with cleavage guided by miR319a/b (Palatnik et al., 2003). To determine whether this constitutes a real but rare event, we developed a rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) assay to distinguish between miR159 and miR319-triggered cleavage products (for details, see Figure S4). This assay confirmed that *MYB33*, which has a target site identical to that of *MYB65*, is indeed occasionally targeted by miR319a in wild-type plants (Figure 3A). MiR319-triggered cleavage products of *MYB33* are more easily detected in strong 35S:*miR319a* overexpressers (Figure S4). A transgene reporting expression of the *MIR319a* primary transcript revealed transient expression in young floral buds (Figure S5), in which the *MYB33* promoter is active as well (Millar and Gubler, 2005). Thus, there is at least some overlap of miR319 and *MYB33* expression, although miR159 is expressed much more widely and more strongly than miR319. Furthermore, the *MYB33* and *MYB65* promoters are strongly active in many tissues and stages in which we could not detect *MIR319a* promoter activity, thus indicating that both differences in expression level and pattern contribute to the preferred targeting of *MYB* mRNAs by miR159.

The rare cross-regulation of *MYB* targets by miR319a/b raised the question of whether it is simply impossible to generate a *TCP*-specific miRNA or whether cross-regulation is tolerated by the plant as an infrequent and therefore largely innocuous event. Using the Web MicroRNA Designer (WMD) tool (Schwab et al., 2006), we designed two miR319a variants that should retain their activity toward the *TCPs* at the expense of interaction with the *MYBs*. Changes at positions 14, 15, and 17 were sufficient to strongly reduce the ability of miR319 to cause male sterility, indicative of reduced *MYB* targeting, while retaining the ability to induce altered leaf morphology, indicative of normal *TCP* targeting (Figure 3C). Additional modification of positions 19 and 20 did not further modify miR319a overexpression phenotype (Figure 3D).

In contrast to cross-regulation of *MYB* genes by miR319a, computational predictions, cleavage site mapping, and overexpression phenotypes suggest that despite their high expression levels, miR159a and miR159b have no effect on *TCP* genes (Achard et al., 2004; Millar and Gubler, 2005; Palatnik et al., 2003) (Figure 1). We were unable to obtain plants overexpressing miR159c, which can potentially target two *TCP* genes (Figure 1D), although we detected expression of the *MIR159c* precursor in 35S:*miR159c* transgenic lines (Figure S6). In addition, we did not detect an increase in miR159c levels when the 35S:*miR159c* construct was transiently introduced into *N. benthamiana* leaves (Figure 4A). It therefore appears that the low miR159c levels in normal plants are at least partially due to inefficient processing. Together, these findings suggest that the potential effect of miR159c on *TCP*s is not relevant in vivo, consistent with the observation that we have never seen a cleavage product indicative of *TCP* targeting by miR159c.

A shortcoming of the evidence presented so far is that we could not exclude translational effects of miR159a/b on *TCP*s, similar to the translational inhibition caused by miRNAs, with limited sequence complementarity to their targets in animals. To analyze translational repression by

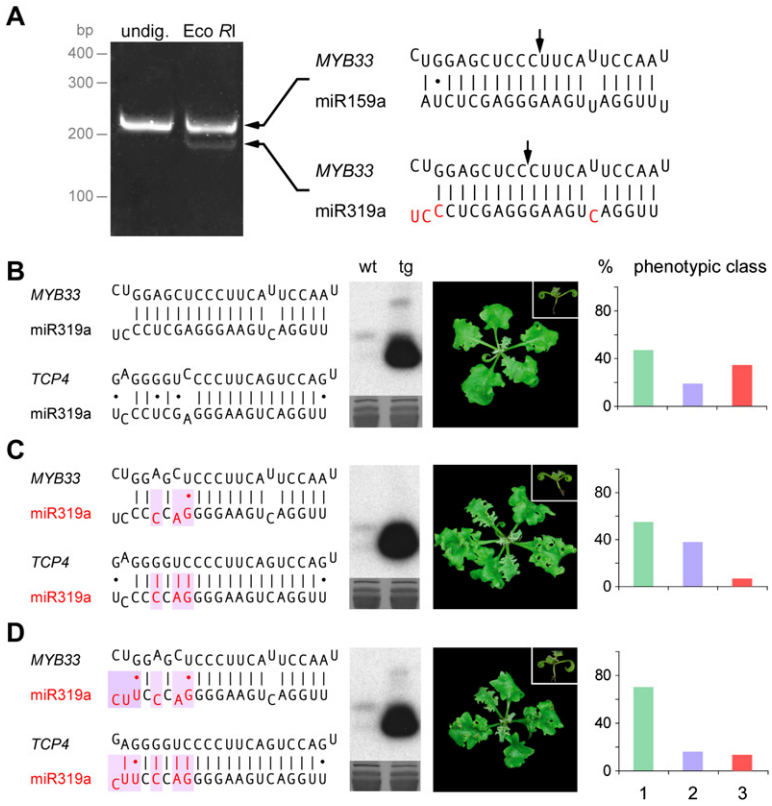

Figure 3. Targeting of *MYB33* by miR319a

(A) Detection of miR159a/b- and miR319a/b-guided cleavage fragments of *MYB33* in wild-type plants by a modified 5′ RACE-PCR assay (for details, see Figure S4). PCR products before (undig.) and after diagnostic digestion with Eco *RI* restriction enzyme are shown.

(B–D) Left, alignment of *TCP4* and *MYB* target sites with miR319 variants (mutations indicated in red). Middle, small RNA blots (probed for miR319a and variants in wild-type [wt] and transgenic [tg] plants) and rosettes of miRNA overexpressers. Right, distribution of phenotypes, assessed by number of fruits that produced seeds on individual plants (1, over 50% of fruits, or most like wild-type; 2, between 10% and 50% of fruits; 3, less than 10% of fruits, or most like miR159 overexpressers). Phenotypic class 3 is most like that of miR159 overexpressers due to male sterility (Figure 2A). In variant (D), position 21 was also changed so that it would now perfectly match *TCP4*, if a 21 nucleotide long version of miR319a were made.

miRNAs, we transiently expressed a TCP4:GFP fusion protein in *N. benthamiana* leaves using *A. tumefaciens*. As expected, coexpression of miR319a led to strong reduction of *TCP4:GFP* transcript levels and GFP fluorescence (Figures 4A and 4B). Even the weaker expression of miR319b (which is identical to miR319a) from a 35S:*miR319b* construct had a pronounced effect on *TCP4:GFP* RNA levels (Figure 4B). As a control, we prepared a version of *TCP4:GFP* with multiple mutations in the miRNA complementary motif. MiR319 coexpression had no effect on mTCP4:GFP RNA or protein (Figures 4A and 4B). We further tested susceptibility of *TCP4:GFP* mRNA to miR319a in a semiquantitative fashion. Even after coinfiltration with a 100-fold dilution of the 35S:*miR319a* vector, we detected some degradation of *TCP4:GFP* mRNA, indicating exquisite sensitivity of *TCP4* to miR319-guided cleavage (Figure S7).

Although endogenous levels of miR159 are substantial in *N. benthamiana* leaves (Figure 4A), *TCP4:GFP* mRNA and fluorescence are easily detected when *N. benthamiana* leaves are not coinfiltrated with a 35S:*miR319* construct, indicating that miR159 affects neither *TCP4:GFP* mRNA nor protein. We attempted to further increase miR159 levels by coinfiltrating 35S:*miR159a* or 35S:*miR159b* constructs, but no obvious effects on mRNA levels or GFP fluorescence were seen (Figure 4B). These findings are in agreement with the observation that miR159 overexpression in *Arabidopsis* does not cause epinastic cotyledons or crinkly leaves, which would be the consequences of *TCP* inactivation.

Basis of miR159 Specificity

All our experiments indicated that miR159 specificity is encoded in the miRNA itself. Further support for this comes from ^{1}H nuclear magnetic resonance (NMR) spectroscopy, which revealed differences in the in vitro structures of miR159-*MYB33* and miR159-*TCP4* RNA duplices (Figure S8). To identify nucleotide positions that underlie the differential interaction of miR159a with *MYB* and *TCP* mRNAs in vivo, we turned to site-directed mutagenesis. Mutant miRNA variants were expressed in the context of a genomic *MIR159a* fragment, which had no effects on leaf morphogenesis in its wild-type form (Figure 5A). As a control, we expressed the *MIR319a* fold-back under control of *MIR159a* genomic sequences. The strongly crinkled leaves and epinastic cotyledons caused by this transgene (Figure 5G), similar to those seen in 35S:*miR319a* plants, indicated that the much higher levels of miR159a in plants compared to those of miR319 are at least partially due to broader and more extensive activity of *MIR159a* regulatory sequences. The results also confirmed that despite their overlapping expression, *TCP*s are normally not targeted by miR159.

At a difference with 35S:*miR319a* plants, the expression of miR319a from the *MIR159a* regulatory sequences did not induce male sterility, which is consistent with limited expression of miR159 in tissues where *MYB* activity is required. Thus, miR319a-like activity of miR159a variants should be detectable without ectopic expression. Nevertheless, we tested the mutant precursors also under the control of the 35S promoter to account for potentially

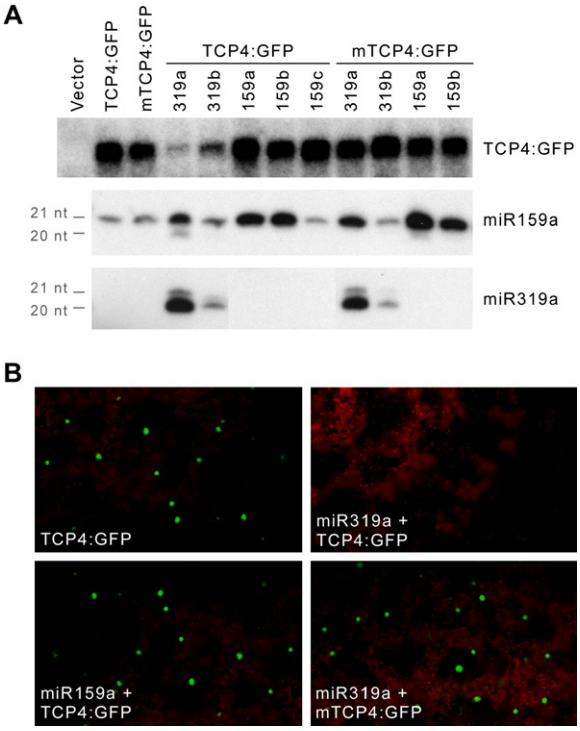

Figure 4. Specific Regulation of *TCP*s by miR319

(A) Effects of different miRNAs on *TCP4:GFP* RNA level in *N. benthamiana* transient assay.

(B) Effects of different miRNAs on TCP4:GFP fluorescence in *N. benthamiana* leaf epidermal cells.

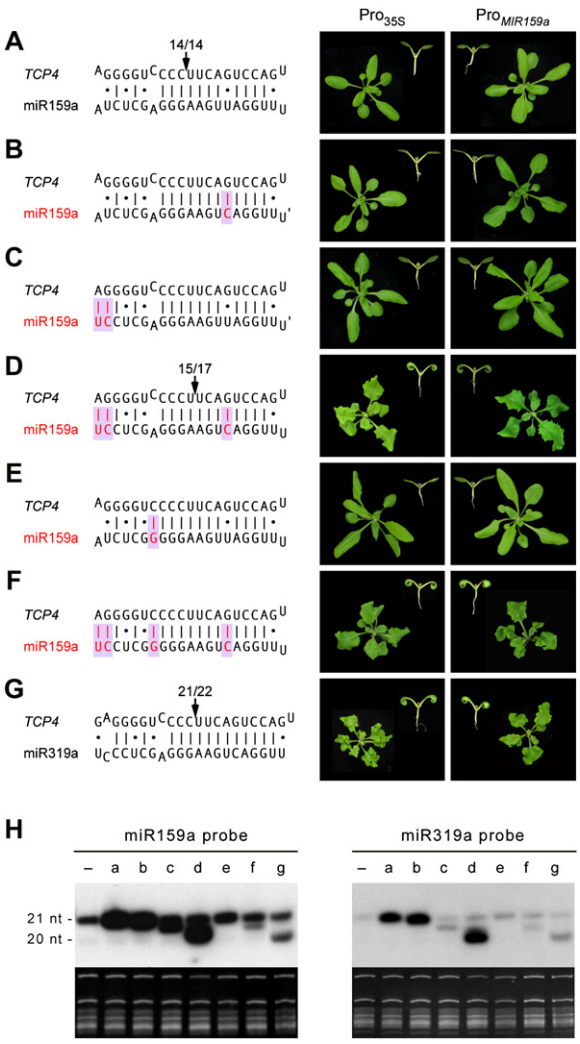

Figure 5. Structure-Function Analysis of miR159a

(A–G) Left, alignment between *TCP4* target site and miRNAs (mutations are indicated in red). *TCP4* cleavage site is indicated by an arrow where experimentally determined. Right, rosette and seedling phenotypes caused by expressing the miRNA precursors under control of the 35S promoter or miR159a regulatory regions. At least 50 independent T_1 plants per construct were analyzed. When observed, epinastic cotyledons were present in more than 80% of T_1 plants, while crinkled leaves were present in at least 60%. An exception was the miR159a genomic construct (D), which produced epinastic cotyledons and crinkled leaves in greater than 60% and 40% of T_1 plants, respectively. In the case of (B) and (E), T_1 plants were similar to plants expressing wild-type miR159a. Arrows indicate the cleavage products identified in RACE libraries made from transgenic plants. Note that the position is shifted by one nucleotide for the mutant miR159 in (D).

(H) Small RNA blots. Inflorescences from 12 independent T_1 plants were pooled irrespective of their phenotypes and analyzed for miRNA expression. (A–G) refers to constructs shown above.

lower expression of the mutant miRNAs. As for the miR319a variants, compensatory mutations were introduced in the miR159a* sequence to maintain the secondary structure of the precursor (Figure S9). All of the mutant miRNAs accumulated in transgenic plants, as shown with small RNA blots, although there were differences in signal intensity. While some of this might be due to variation in miRNA levels, experiments with synthetic miRNAs showed that mutant miRNAs also hybridize less efficiently to the standard miR159a probe (Figure 5H and Figure S10). In addition, some of the mutant miRNAs migrated differently from endogenous miR159a, which is probably caused by distinct secondary structures of the miRNAs (Figure S10).

We introduced point mutations to make miR159a successively more similar to miR319a. Converting the G:U pair at position 7 to a perfect match did not enable miR159a to affect leaf morphogenesis through *TCP* targeting (Figure 5B). That a match at this position alone is neither essential nor sufficient for miRNA activity can also be inferred from validated miR159a targets, such as *MYB33* and *MYB65*, which contain a complete mismatch at position 7 (Figure 1E).

We next mutated the 3′ end of the miR159a sequence, eliminating two mismatches to *TCP4* (because of a different sequence at the extreme 3′ end, this would correspond only to a single change in the case of miR159b). Again, the resulting miRNA triggered no miR319a-like effects on leaf morphology (Figure 5C). However, when the 5′ and 3′ mutations were combined in a doubly mutant miRNA, leaf morphology defects typical for miR319a were seen (Figure 5D). Expression of this and other mutant

forms of miR159a from the 35S viral promoter or its own regulatory region gave similar results, indicating that the observed phenotypes were not a consequence of artificially high levels of the miRNA (Figures 5A–5F). In summary, differences in at least two nucleotide positions insulate the *TCP*s from miR159 targeting.

We also introduced a mutation that corrects a mismatch of miR159a to *TCP4* at position 15. Mutating only this position had no effect on leaf morphology (Figure 5E). The same mismatch occurs in the miR319a:*TCP4* duplex, and this has been conserved during evolution. To determine whether the mismatch is important for *TCP4* targeting, we combined the mutation at position 15, which introduces an additional match, with the previously tested 5′ and 3′ mutations. The altered base pair did not adversely affect the ability of multiply-mutant miR159a to target *TCP4*. If there was an effect at all, this mutant miRNA was even more effective in causing miR319-like effects than the doubly-mutant one (Figure 5F), suggesting that greater complementarity positively affects targeting ability. This is further supported by overexpression of a mutant miR159a in which a mismatch to *MYB33* and *MYB65* at position 7 (see Figure 1E) had been corrected. With this mutant, the number of sterile T1 increased from 48% to 75%, as compared to wild-type miR159a overexpressers.

As mentioned above, the different 5′ ends of miR159a and miR319a allow the identification of the miRNA-executing *TCP4* cleavage in plants (see Figure 1E). To confirm that the mutant miR159a was indeed targeting *TCP4* mRNA, we prepared 5′ RACE libraries from transgenic plants. As expected, in wild-type, 35S:*miR319a*, or MIR159a:*miR319a* plants, the cleavage site is always indicative of miR319a/b guiding *TCP4* cleavage (arrow in Figure 5G). When we examined transgenic plants expressing the unmodified form of miR159a, we found that *TCP4* was still guided to cleavage only by miR319a (Figure 5A), confirming the inability of miR159a to target *TCP4*. On the contrary, when we analyzed plants expressing a mutant form of miR159a, in which the G:U at position 7 and the 3′ end of the miRNA had been changed, the predominant *TCP4* cleavage site was shifted by one nucleotide, consistent with *TCP* degradation triggered by the highly expressed mutant miR159a (Figure 5D).

Suppression of miR319a Overexpression Effects by Point Mutations in a Single Target

In parallel with transgenic expression of mutant miRNAs, we undertook a suppressor screen of *jaw*-D plants, which overexpress miR319a from the endogenous locus (Palatnik et al., 2003) to identify sequences in miR319a or its targets that are critical for miRNA-target interaction. From a genome-wide mutagenesis screen with ethyl methanesulfonate, we isolated 16 *soj* (*suppressor of jaw-D*) mutants with attenuated leaf phenotypes. As a prescreen for the possible defect in *soj* plants, the mRNA levels of each of the five *TCP* targets of miR319a were measured by RT-PCR. Surprisingly, four *soj* mutants showed a specific recovery of only one *TCP* target, *TCP4* (Figure 6A). Sequencing of the genomic *TCP4* locus revealed that all

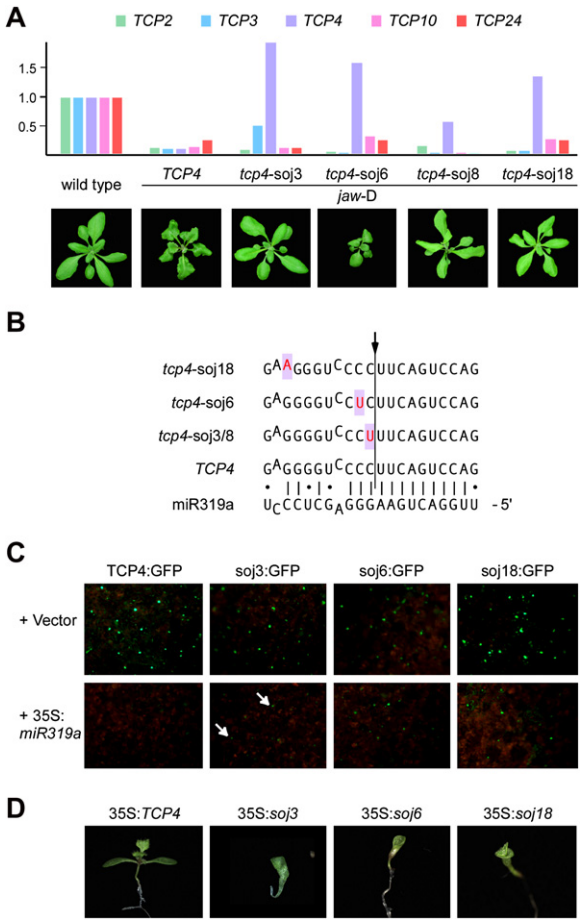

Figure 6. Suppression of the *jaw*-D Phenotype by Mutations at the Endogenous *TCP4* Locus

(A) Relative expression levels of *TCP* mRNAs in *soj* mutants. RNA expression was measured by real-time RT-PCR in the primary screen, as a prescreen for mutants with altered *TCP* levels. Variation in the effects on *TCP4* expression in soj3 and soj8, which carry the same mutation in *TCP4*, are likely due to RNA having been isolated from plants grown at different time points. Images of mutant rosettes shown below.

(B) *TCP4* genomic sequence in *soj* mutants. The cleavage site in wild-type is indicated.

(C) Transient assay in *N. benthamiana* showing that *tcp4*-soj alleles are less susceptible to miR319a-guided cleavage than wild-type *TCP4* but are not completely resistant to miR319a either. White arrows indicate weak GFP signal in the nuclei of transformed cells.

(D) Phenotypes caused by overexpression of *tcp4*-soj alleles. GFP fusions identical to the ones used in (C) were used.

four lines had point mutations in the miRNA complementary motif. *Tcp4*-soj3 and *tcp4*-soj8 had the same mutation corresponding to the nucleotide opposite position 11 of miR319a and thus immediately adjacent to the cleavage site (Figure 6B). *Tcp4*-soj6 had a mutation next to the one in *tcp4*-soj3 and *tcp4*-soj8, at position 12 (Figure 6B). Being close to the cleavage site, it was not surprising that these mutations affected miRNA targeting. A more remarkable and unexpected suppressor mutation was *tcp4*-soj18, which affects pairing with nucleotide 19 at the 3′ end of the miRNA and which, therefore, should

have only a subtle effect on miRNA targeting. However, the effects of all three mutations on the predicted free energy, ΔG, was very similar: −32.8 (soj-3/8), −32.4 (soj-6), and −32.1 (soj-18) kcal/mol. Interestingly, this is in the same range as the free energy of the miR159/*TCP4* duplex, −32.0 kcal/mol.

We had not anticipated the recovery of *tcp4* alleles with mutations in the miRNA target site, because rendering *TCP4* mRNA resistant to miR319 often causes seedling lethality (Palatnik et al., 2003). These apparent contradictions can be reconciled by proposing that the *soj* mutations in *TCP4* only partially affect miRNA-guided target cleavage. To test this hypothesis, we first used the *N. benthamiana* assay to express coding sequences of *tcp4*-soj linked to those for GFP. Coexpression of the fusion constructs with an miR319a-overproducing construct reduced but did not abolish GFP fluorescence (Figure 6C). Thus, the *soj* mutations must be permissive for a degree of miR319a-guided cleavage (or translation inhibition) that is compatible with near-normal development of *Arabidopsis* plants. To demonstrate directly that a combination of changes in expression level and target motif complementarity can shift the balance between *TCP4* stability and targeting, we overexpressed the GFP fusions of the *tcp4*-soj alleles in *Arabidopsis*. In contrast to seedlings that had the *soj* mutations at the endogenous *TCP4* locus, these plants often showed severe patterning defects (Figure 6D) similar to plants with a fully nontargeted version of *TCP4* (Palatnik et al., 2003).

DISCUSSION

We used two major approaches to investigate the differential in vivo effects of two closely related miRNAs. First, we engineered mutations in miR159a and miR319a that alter their differential affinity toward *MYB* and *TCP* targets. Second, we isolated altered alleles of a target locus after random mutagenesis, screening for mutations that can suppress the effects of miR319a overexpression. Importantly, changes in either the miRNA or the target had similar effects on miRNA-target interactions, indicating that the observed effects are not primarily due to different loading of miRNAs onto RISC.

Mechanistic aspects of miRNA-target interaction in *Arabidopsis* have been previously studied by Mallory and colleagues (2004b) using miR165/166 and their target *PHB*. A systematic mutation scan revealed that target mismatches between positions 3 and 11 of the miRNA produced at least a 10-fold decrease in miRNA-guided cleavage activity, while mismatches at the 3′ end had a much smaller effect. Subsequent work by Schwab and colleagues (2005), who analyzed global transcript profiles of several miRNA overexpressers, not only confirmed the importance of the 5′ region of the miRNAs but also highlighted significant contributions of the 3′ region to target recognition.

All three mutations in *TCP4* that suppress the effects of miR319a overexpression have small effects on the calculated free energy. Although the soj3/8 and soj6 mutations map near the cleavage site, they replace perfect matches with G:U base pairs. They therefore support the notion that a G:U pair cannot necessarily replace a G:C canonical interaction in miRNA-target recognition in plants. The soj18 mutation, which affects pairing with miR319 at position 19, also causes only a small difference in the calculated interaction free energy. Previous work has shown that substitutions in the 3′ region of the target site have clear effects on in vitro cleavage, but the phenotypic effects appear to be small (Mallory et al., 2004b). An exception is a mutation in the maize gene *rolled leaf1* at position 18 of the miRNA target site, which interferes with miR166-triggered transcript cleavage (Juarez et al., 2004). The importance of the 3′ end in addition to pairing of the 5′ region is in agreement with a recent report that animal siRNAs can effectively distinguish between nearly identical targets containing one difference at position 16 (Schwarz et al., 2006). The particular sensitivity of the miR319-*TCP4* duplex to mutations that affect pairing of the 3′ region of the miRNA may be due to this duplex having more mismatches than other plant miRNAs and their targets. When *tcp4*-soj18 is aligned with miR319a, there are only two canonical base pairs among the last seven nucleotides, with two additional G:U wobbles. The picture that emerges then is that the interaction between the miRNA and the target should be analyzed in the usual sequence context. In animals, the context of the target site can influence the final outcome of the miRNA-target interaction (Didiano and Hobert, 2006).

Members of the miR159 and miR319 miRNA families are closely related in sequence, but it has been unclear whether they also overlap in function. Mapping of cleavage sites in wild-type plants has not provided any evidence for miR159 targeting the *TCP* genes (Palatnik et al., 2003), and experimentally established criteria for miRNA-guided cleavage clearly argue against *TCP* targeting by miR159 (Schwab et al., 2005). Our mutational approach has shown that changes at three nucleotide positions, in the 5′ seed region and at the 3′ end, are sufficient to allow effective cleavage of *TCP*s guided by miR159a (Figure 5). Because of a sequence difference at the last position, this would correspond to only two altered nucleotides in the case of miR159b. A mutational approach was also taken by Brennecke and colleagues (2005) to analyze target recognition determinants. They identified three target classes for *Drosophila* miRNAs, termed 5′ dominant, 5′ seed, and 3′ compensatory, reflecting that miRNA recognition of targets can rely on different mechanisms. These authors found that reporters for the targets *grim* and *sickle* were differentially regulated by the miRNAs miR-2, miR-6, and miR-11. The divergent 3′ regions of these miRNAs are apparently responsible for target selection, although the exact nucleotides involved were not identified.

It has been a matter of debate how far translational repression needs to be taken into account when assessing plant miRNA effects. In at least one case, miR172, both cleavage and translational repression are used as regulatory mechanisms, although the effects of cleavage can be obscured by a transcriptional feedback loop (Aukerman

and Sakai, 2003; Chen, 2004; Lauter et al., 2005; Schwab et al., 2005). There is no evidence for miR159 affecting protein levels of *TCP* targets, as concluded both from phenotypic and molecular differences between miR159 and miR319 overexpressers in *Arabidopsis* (Achard et al., 2004; Millar and Gubler, 2005; Palatnik et al., 2003), and from a transient assay monitoring effects on target RNA and protein in *N. benthamiana* (Figure 4). The absence of major translational effects on potential targets with limited sequence complementarity is supported by the close phenotypic and molecular similarity of plants overexpressing natural or artificial miRNAs with plants that have mutations in the miRNA target genes (Alvarez et al., 2006; Aukerman and Sakai, 2003; Chen, 2004; Laufs et al., 2004; Mallory et al., 2004a; Schwab et al., 2006; Schwab et al., 2005). Together, these findings support the hypothesis that the sequence requirements for transcript cleavage and translational inhibition in plants are similar.

In contrast to predominant targeting of *TCP* genes by miR319, statistical as well as experimentally established criteria have suggested that miR319 should be as efficient in targeting *MYB33* and *MYB65* as miR159 (Jones-Rhoades and Bartel, 2004; Mallory et al., 2004b; Schwab et al., 2005) (Figure 1E). However, since miR159 is much more abundant than miR319 (Table 1), miR319 does not normally make an important contribution to *MYB* regulation, which explains why *MYB* degradation products indicative of miR319a/b-guided cleavage are rare in wild-type plants but can easily be detected in *miR319a* overexpressers (Figure S4). Reporter studies indicate that *MYB33* and *MYB65* transcription occurs widely in many tissues, and it is the miRNA activity that restricts the final pattern of expression to a much narrower domain (Millar and Gubler, 2005). The high rate of *MYB33* and *MYB65* turnover throughout most tissues impedes the detection of any additional cleavage events that can be caused by miR319 in the few cells in which *MYB33* and *MYB65* escape miR159 targeting.

In conclusion, the apparent specificity of miR319 for regulation of *TCP*s and of miR159 for *MYB*s is achieved through different mechanisms. The predominant targeting of *MYB*s by miR159 rather than miR319 appears to be simply due to much higher levels of miR159, while sequence differences ensure that the much more abundant miR159 does not target *TCP* mRNAs, which can only be guided to cleavage by miR319. The importance of relative expression of targets and miRNAs is also supported by our finding that overexpression of *tcp4*-soj alleles causes severe defects, while expression of these alleles from the endogenous locus has only limited adverse effects on plant development.

Our work leads to two sets of questions. The first relates to the possibility that the more specific miR159 evolved from miR319, which can target both *TCP*s and *MYB*s. Although there is some sequence similarity between the miR159 and miR319 precursors outside the miRNA and miRNA* sequences, these are not significantly higher than what is seen for some other pairs of apparently unrelated miRNAs (Figure S2). So far, however, only miR319 has been isolated from the moss *P. patens* (Arazi et al., 2005; Talmor-Neiman et al., 2006), from which seed plants including *Arabidopsis* diverged some 300 million years ago. The impending completion of the genomes of *Physcomitrella* and other nonseed plants may answer the question of miR159 origin.

The second, more general, question concerns the role of sequence differences in subfunctionalization of plant miRNAs. For transcription factors, it is widely accepted that both tissue-specific expression patterns and differences in DNA binding specificity contribute to subfunctionalization among different members of the same family. In animals, there is precedence for subfunctionalization among miRNA family members based on sequence variation (Abbott et al., 2005; Brennecke et al., 2005). In plants, few efforts have been made to distinguish between different targets of miRNA families, with the exception of the miR164 family, for which differential in vivo effects due to expression differences have been demonstrated (Baker et al., 2005; Guo et al., 2005; Nikovics et al., 2006; Sieber et al., 2007). Our results indicate that sequence changes at two nucleotide positions suffice to widen miR159b specificity from one to two target families. Sequence analysis of conserved miRNA families in *Arabidopsis* reveals that 30% vary at two nucleotide positions or more between members (Table S4). This suggests additional opportunities for sequence-based miRNA subfunctionalization, which might cause individual miRNA family members to preferentially affect different mRNAs within a related family of targets. It will be important to determine the broader role of subfunctionalization due to expression and sequence differences in the evolution of RNA-based regulatory networks.

EXPERIMENTAL PROCEDURES

Plant Material

Arabidopsis ecotype Col-0 was used for all experiments. Plants were grown in long days (16 hr light/8 hr dark) or in continuous light at 23°C.

Transgenes

See Table S2 for a list of binary plasmids used in this study. Mutated versions of miR159a/miR159a*, miR319a/miR319a*, and *DUO1* were generated by PCR. The miR159a genomic construct comprised nucleotides 27,717,099 to 27,718,771 of *Arabidopsis* chromosome 1. A 1.3 kb fragment upstream of miR319a (position 1 to 1328 in accession number AY922326) was used to monitor *MIR319a* promoter activity.

Small RNA Analysis

RNA was extracted using Trizol reagent (Invitrogen), the Plant RNeasy Mini kit (QIAGEN), or the mirVANA miRNA Isolation Kit (Ambion). Total RNA was resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). A total of 4 fmol synthetic RNA oligonucleotides (Biomers) were used as size standards. Blots were hybridized using radioactively end-labeled LNA oligonucleotide probes designed against miR159a or miR319a (Exiqon, Denmark) or conventional DNA oligonucleotides.

Cleavage Site Mapping of miRNA Targets

Poly(A)$^+$ RNA was extracted from 50 μg of total RNA with the Oligotex Mini kit (QIAGEN). Ligation of an RNA adaptor, reverse transcription, and 5′ RACE-PCR were done according to manufacturer's instructions

for the GeneRacer kit (Invitrogen). Two nested gene-specific reverse oligonucleotides were used for RACE (Llave et al., 2002). A modification of the technique was used to differentiate between miR319a/b-specific and miR159a/b-specific cleavage. Briefly, the second nested generic oligonucleotide from the GeneRacer kit was modified such that it will generate an Eco *R*I restriction site on miR319a/b-specific products. Differential cleavage by miR319a/b versus miR159a/b was detected by restriction analysis and polyacrylamide gel electrophoresis using SYBR Green as stain (see Figure S4 for details).

Real-Time RT-PCR

First-strand cDNA synthesis was done with 1–2 μg total RNA using the Cloned AMV First-Strand Synthesis Kit (Invitrogen) or the RevertAid First Strand cDNA Synthesis Kit (Fermentas). ABsolute QPCR SYBR Green Mix (Invitrogen) was used in the real-time PCR. Amplification was monitored with the Opticon Continuous Fluorescence Detection System (MJR). Primer sequences are shown in Table S3.

Transient Expression in *N. benthamiana*

N. benthamiana leaves were transfected as described (Llave et al., 2002). The *A. tumefaciens* strains harboring the miRNA overexpression constructs were used in 4-fold excess over the strains with the target constructs, unless stated otherwise. GFP fluorescence was imaged 3 days after coinfiltration.

Supplemental Data

Supplemental Data include ten figures and four tables and can be found with this article online at http://www.developmentalcell.org/cgi/content/full/13/1/115/DC1/.

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